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CHEMISTRY OF THE LICHEN *Hypogymnia physodes* TRANSPLANTED TO AN INDUSTRIAL REGION

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Abstract—Lichens produce a great number of secondary metabolites that participate in ecological interactions and respond to environmental changes. We examined the influence of heavy metal accumulations on lichen secondary metabolism. Thalli of *Hypogymnia physodes* were transplanted for 6 months to the Cracow–Silesia industrial region. Based on heavy metal accumulations in lichen, two of the investigated sites were classified as highly polluted. The highest concentrations of Cd, Pb, and Zn were found in lichens transplanted in the vicinity of a Zn–Pb smelter. Significant accumulations of Cr and Ni were detected in *Hypogymnia* transplanted near a chemical industry. Physodic, physodalic, hydroxyphysodic acids, and atranorin were identified and analyzed in extracts obtained from specimen samples. The most detrimental changes were observed in lichen transplanted into the vicinity of a chemical industry producing chromium, phosphor, and sulfur compounds that contained 340-fold higher Cr levels than control thalli. Decreases in the levels of physodic acid, hydroxyphysodic acid, and atranorin were detected, and one additional polar compound (probably product of degradation of lichen acids) appeared in the extract. The content of physodalic acid increased in every thalli sample transplanted, suggesting a possible role of this compound in defense against stress caused by accumulated pollutants. The levels of physodic acid decreased in thalli from both of the most polluted sites compared to those of the controls—but were not changed in thalli transplanted to less polluted sites. Our results illustrate that lichen compounds are sensitive to heavy metal accumulation and could be used as biomarkers in environmental studies.

Key Words—Air pollution, heavy metals, sulfur, secondary metabolites, biomarkers.

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INTRODUCTION

Lichens are the symbiotic association between a fungus and a photosynthetic partner such as an alga or cyanobacterium. They are perennial and maintain a uniform morphology over time. They grow slowly, have a large-scale dependence on the environment for nutrition, and do not shed parts during growth. The lack of epidermis, stomata, and cuticular waxes makes these organisms incapable of controlling gas exchange and allows absorption of

can act as antifeedants for insects and other animals (Clark et al., 1999; Huneck, 1999; Molina et al., 2003). Lichens typically grow under extreme conditions of temperature, humidity, intensity of light, or excessive exposure to heavy metals. The Acarosporetum was found to be the most metal-resistant lichen community (Beck, 1999). Some lichen metabolites are considered to be stress metabolites synthesized in response to biotic and abiotic stimuli. These substances may protect thalli from the dangerous toxic actions of free radicals produced by oxidative stress exposure (Huneck and Yoshimura, 1996; Caviglia et al., 2001).

Lichen secondary metabolites have been investigated mostly for chemotaxonomic purposes and in connection with their potential as phytomedicines and natural biopesticides (Huneck and Yoshimura, 1996; Peres and Nagem, 1997; Huneck, 1999; Dayan and Romagni, 2001; Manojlovic et al., 2002). Only a few studies have considered changes in lichen secondary metabolites caused by environmental stress (Calatayud et al., 2000; Caviglia et al., 2001; Conti and Cecchetti, 2001; MacGillivray and Helleur, 2001). Because these compounds may play a role in the adaptation of lichens to their environment, as well as in ecological interactions, they are suitable candidates for the detection of detrimental changes in ecosystems caused by contamination (Jezierski et al., 1999; Caviglia et al., 2001).

A given stressor might cause an increase in the production of a lichen acid, or the normal level of lichen acids might be reduced as they react or decompose in response to stress (MacGillivray and Helleur, 2001). Because the roles of lichen secondary metabolites have not been well established, lichen transplantation seems to be a relevant model system for such investigations. Moreover, lichen compounds in thalli transplanted to polluted areas have not been studied so far.

The goal of the present investigation was to establish the accumulation of metals and sulfur as well as a number of selected secondary metabolites profile in lichen *H. physodes* (L.) Nyl. transplanted to sites with different pollution exposure.

METHODS AND MATERIALS

Test Sites. The studies covered the Cracow–Silesia industrial region in southern Poland (Figure 1). This area has a population density of ca. 280 persons/km. It is one of the most polluted areas in Europe, and still has among the highest emissions of heavy metals in Poland. The investigated sites were located in the vicinity of major emission sources from industrial plants, all of which have been placed on the list of industries with the most harmful impact on the environment in Poland (Bereś et al., 2003). As a northwesterly wind predominates in this region of Poland, the locations of the test sites were selected

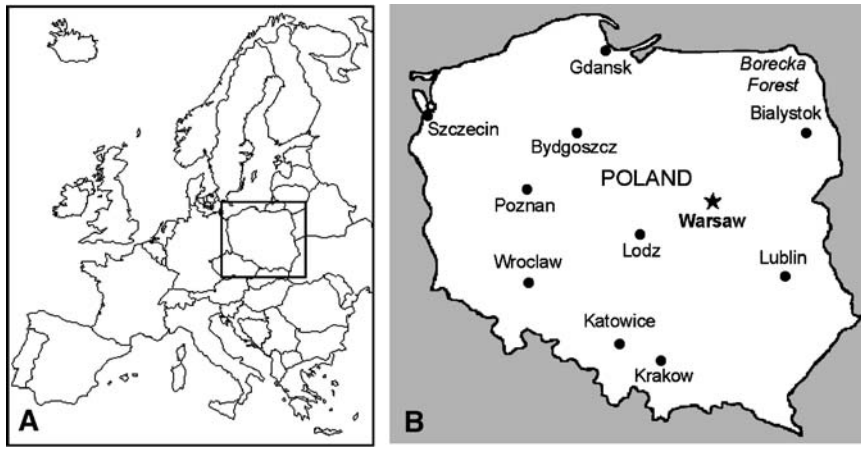

FIG. 1. A) The geographical location of Poland within Europe. B) Major cities in Poland, including the location of the unpolluted Borecka forest where the original lichen samples were collected (northeast of Poland), and the Cracow region where the tests were conducted.

southeast of the emission sources. The following sites of area 200 m^2 were chosen for the study:

1. Bukowno (Figure 2). This site is in the immediate vicinity of the "Bolesław^ Zn–Pb smelter. This industry was founded in 1949 and mainly involves mining of zinc and lead, as well as production of their concentrates. In 1990, this industry emitted over 17, 7, and 0.3 tons of Zn, Pb, and Cd, respectively. Since then, successively lower emission levels have been observed. In 2000, emission of particulates reached 0.753, 0.0022, and 0.0002 tons of Zn, Pb, and Cd, respectively (data provided by the Zn–Pb smelter).
2. Młoszowa (Figure 2). The main pollution source in this site (the Trzebinia S.A. refinery) was established at the end of the 19th century. It produces fuels, asphalts, paraffin, and oils. The quality of the environment at this site may also be affected by emission from the "Siersza^ S.A. power plant in Trzebinia. This study area is also relatively close to the A-4 Cracow–Katowice highway.
3. Jankowice (Figure 2). This site is affected by emission from the chemical industry "Dwory^ S.A. in Oświęcim. This industry emitted 743,000 tons of gaseous pollutants (mainly PAHs, Cl, and S compounds) in 2000 (Bereś et al., 2003).
4. Alwernia (Figure 2). This site is located in the immediate vicinity of the "Alwernia^ S.A chemical industry, which was established in 1924.

Currently, the industry produces chromium, phosphorus, and sulfur compounds. Emission of dusts from "Alwernia^ in 2000 reached 78,000 tons, including 1.7 tons of Cr. In 2002, the industry emitted 1.3 tons of Cr and 263 tons of gaseous pollutants (data provided by "Alwernia^ S.A.).

Lichen Transplantation. Control lichens for transplantation were collected in the Borecka Forest located in the northeastern part of Poland (Figure 1), far from large metropolitan areas or other pollution emission sources (Śnieżek, 1997). Samples of the epiphytic foliose lichen *H. physodes* (L.) Nyl., growing on branches of hazel tree (*Corylus avellana* L.), were collected and transplanted to the various study sites on April 15, 2002. Ten branches, ca. 30 cm long, containing about 10–15 g wet weight of the thalli in total, were brought to each site and hung on selected trees at a height of ca. 1.5 m (Figure 3). After 6 months, thalli were separated from the bark and analyzed. Untreated controls consisted of samples collected and transplanted within the Borecka Forest.

Elemental Analysis of Thalli Tissues. Thalli collected from the various test locations were oven-dried to constant weight at 60°C and ground in an electric mill. Samples weighing 0.3–0.5 g were digested in $HNO_3/HClO_4$ (4:1) on a water bath at 80°C for about 3 wk (until totally clear). Cd, Cr, Ni, and Pb levels

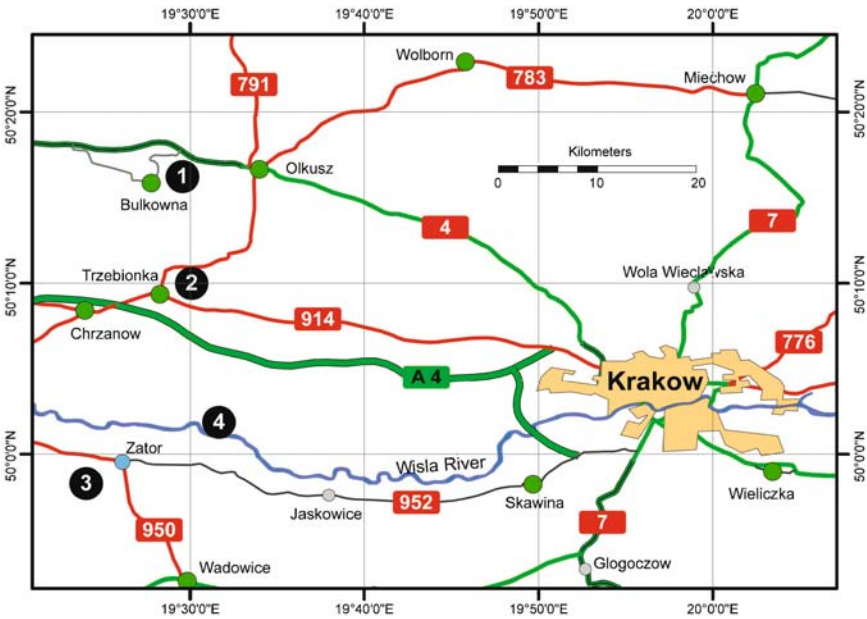

FIG. 2. The Cracow region showing the locations of the various test sites.

FIG. 3. Transplanted lichens hanging from a birch tree.

were determined by using an Aanalyst 800 Perkin-Elmer atomic absorption spectrophotometer with a graphite furnace. Cu, Fe, and Zn concentrations were determined with an IL 251 flame spectrophotometer. Results were expressed in $\mu g\ g^{-1}$ of dry weight. The accuracy of the analysis was checked against standard reference material, namely, Tomato Leaves SRM 1573a.

Total levels of sulfur in transplanted lichens were detected with the turbidimetric method by Butters-Chenry (Nowosielski, 1968). Homogenized thalli (0.2 g) were treated with 75% nitric acid and left for 24 hr at 18°C, evaporated, and treated with magnesium nitrate. This treatment was followed by oxidization at 450°C and treatment with 25% nitric acid, 50% acetic acid, 85% orthophosphoric acid, and 2% barium chloride. The resulting opaqueness was measured by colorimeter (Spectromom 204) at 290 nm. Concentrations of sulfur were calculated according to a $MgSO_4$ standard curve. Results were expressed in $\mu g\ g^{-1}$ of dry weight.

Analysis of Secondary Metabolites in Thalli. After transplantation, visually similar thalli (1.5–2 g of air-dried thalli) were extracted three times for 15 min with 100% ethanol (about 30 ml/g of lichen) with the use of a sonicator. Extracts were filtered and evaporated on a rotary evaporator. Crude extracts (2.5 mg) were diluted in 3 ml of methanol [high-performance liquid chromatography (HPLC) grade]. For separation and analysis of the secondary metabolites, 20 μl of samples were injected onto an analytical HPLC. The HPLC system consisted of a Waters 717 with autosampler, a Waters 600 controller, a Waters photodiode array detector, and 4.6 × 250 mm analytical column (Water Spherisorb 5 μm ODS).

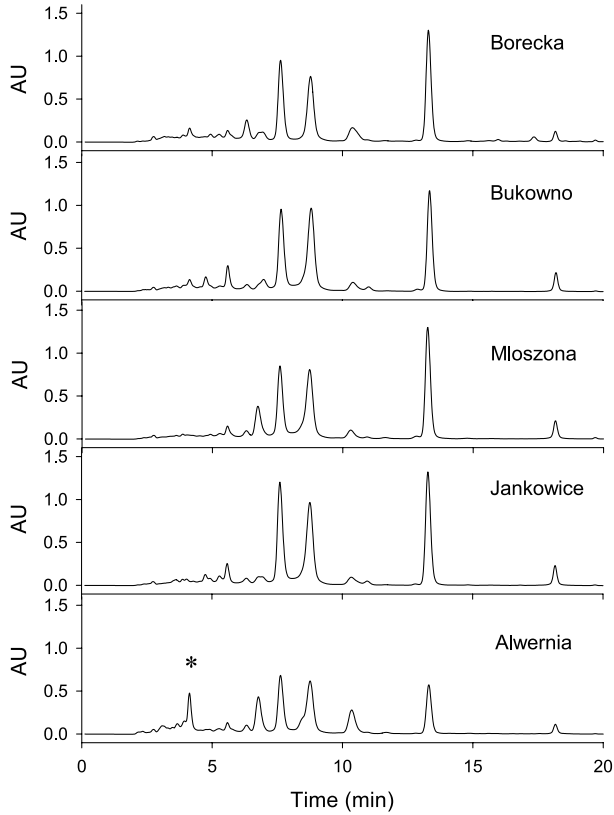

FIG. 4. Chromatograms of crude extracts from *H. physodes* samples from the various test locations. The retention time of hydroxyphysodic acid (7.7), physodalic acid (8.7), physodic acid (13.5), and atranorin (18.1) in the crude extracts matched those of the analytical standards at 7.6, 8.8, 13.3, and 18.2 min, respectively. The identities of the peaks were confirmed by comparing the UV spectra in the crude extract to that of the analytical standards. The polar peak in Alwernia chromatogram is denoted by an asterisk.

The best separation was obtained with 1% phosphoric acid in water (**A**) and methanol (**B**) (Feige et al., 1993, modified) using a gradient from 75 to 100% **B** over 15 min. The flow rate was 1 ml/min and pressure 1500–2000 mm Hg. Elution of the secondary metabolites was monitored at 254 nm. Concentrations of compounds were estimated and/or compared based on peak areas. Compounds were identified by comparison with standards (retention time and UV absorption). Except for the absolute amount, the percentage of total peak area in the whole extract was given. Crude extracts constituted 15–18% of the air-dried weight of thalli. Chromatographic separation revealed three major metabolites (Figure 4) identified as hydroxyphysodic acid (Rt = 7.6 min), physodalic acid (Rt = 8.8 min), and physodic acid (Rt = 13.3 min). Additionally, a smaller peak was recognized as atranorin (Rt = 18.2 min). The chemical structures of these compounds are given in Figure 5. Lichen standards were previously isolated from *H. physodes* at the Center for Research on Natural Products, University of Mississippi. The structures were identified by mass spectrometry and nuclear magnetic resonance. Standard deviations of the HPLC analysis reproducibility were lower than 2.5%.

Statistical Analysis. Means and standard deviations were calculated for elements and secondary compounds. Statistical differences in the concentrations of Cd, Fe, Ni, S, Zn, and lichen acids in thalli transplanted to different sites were established using ANOVA on logarithmic data, followed by Tukey honestly significant difference for equal sample sizes. For determination of statistical differences in Cu, Cr, and Pb concentrations, Kruskal–Wallis nonparametric tests were applied followed by paired comparisons of the mean ranks (Sachs, 1984).

RESULTS

Accumulation of Elements. Six months after transplantation of the lichens, the highest concentrations of Cd, Pb, and Zn were found in samples transplanted to the Bukowno location, in the vicinity of the Zn–Pb smelter, relative to both control thalli and thalli transplanted to the other sites (Table 1). Pb concentrations did not increase in the other locations, relative to the control thalli (about 10 $\mu g\ g^{-1}$). However, Pb reached 124 $\mu g\ g^{-1}$ in lichen transplanted to Bukowno (Table 1). Similarly, Zn accumulation was rather low in all of the studied sites, except for Bukowno where it increased from 55 $\mu g\ g^{-1}$ (control thalli) to 583 $\mu g\ g^{-1}$ (Table 1). Accumulations of Cd were found in all of the transplanted lichens. The level of this element in thalli from Bukowno was ca. 14-fold higher than control thalli. The level increased only 2–2.5-folds in thalli transplanted to the other sites studied (Table 1).

Significant accumulations of Cr and Ni were detected in *H. physodes* transplanted to the Alwernia location (Table 1). The level of Cr 6 months after transplantation to this site was as much as 343-fold higher than in control

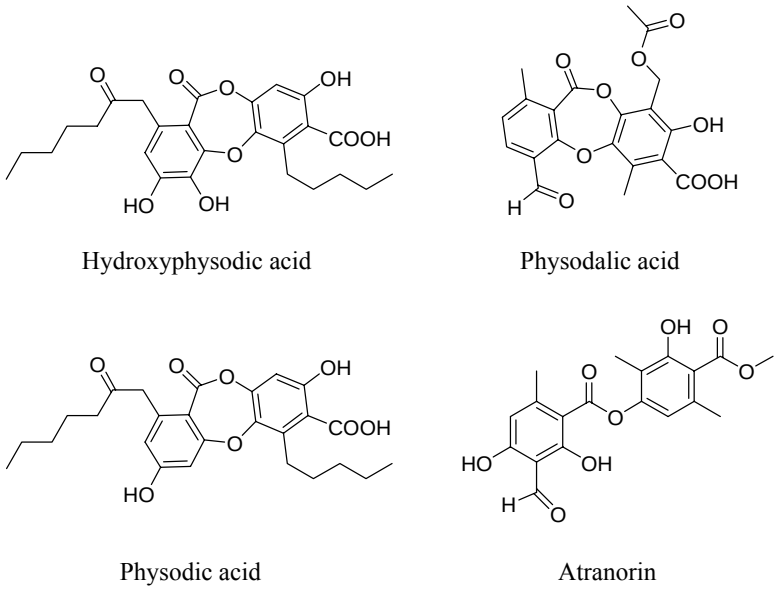

FIG. 5. Structures of the secondary metabolites detected in *H. physodes*.

lichens (Table 1). Ni increased to 9.18 μg g^{-1} compared to 1.08 μg g^{-1} in the control. Additionally, the concentration was slightly increased in thalli from the Bukowno location, but was not statistically different from the rest of the sites (Table 1).

Increases in Cu and Fe concentrations occurred in all thalli after transplantation, but the highest accumulations were noted in *Hypogymnia* samples from Bukowno and Alwernia (Table 1).

Finally, there were significant accumulations of sulfur in all transplanted lichens. Sulfur concentrations ranged from 1406 to 2188 μg g^{-1} in thalli transplanted to the polluted sites compared to 1237 μg g^{-1} in control thalli (Table 1).

Secondary Metabolites in Transplanted Thalli. Significant differences in the quantity of secondary metabolites were found between control thalli from Borecka Forest and thalli transplanted to the polluted areas, as well as among lichens transplanted to areas of different pollution levels.

The most significant decrease in physodic acid (−58% relative to control thalli) was detected in thalli transplanted to Alwernia. The level of physodic acid decreased also in transplanted lichens transplanted in Bukowno (−13% relative to the control thalli). The contents of this compound were not changed in *Hypogymnia* transplanted to Młoszowa or Jankowice (Figure 6A).

TABLE 1. CONCENTRATION OF ELEMENTS IN *Hypogymnia physodes* TRANSPLANTED TO THE CRACOW–SILESIA INDUSTRIAL REGION

Locations	Elements (μg g^{-1} dry weight)							
	Pb	Zn	Cd	Cr	Ni	Cu	Fe	S
Borecka	9.0 ± 0.4a	55 ± 7a	0.54 ± 0.02a	0.22 ± 0.04a	1.08 ± 0.19a	3.7 ± 0.4a	350 ± 47a	1237 ± 33a
Bukowno	123.7 ± 20.0b	583 ± 56c	7.70 ± 0.47d	0.79 ± 0.06b	1.91 ± 0.12b	10.8 ± 0.3d	1306 ± 164c	1842 ± 397b
Młoszowa	11.2 ± 1.5a	68 ± 6ab	1.37 ± 0.05c	0.48 ± 0.06b	1.36 ± 0.19ab	7.5 ± 0.4bc	720 ± 106b	1960 ± 109b
Jankowice	11.3 ± 1.2a	55 ± 4a	1.02 ± 0.12b	2.89 ± 0.54b	1.38 ± 0.06ab	5.9 ± 0.3b	597 ± 38b	1999 ± 242b
Alwernia	12.7 ± 3.1a	77 ± 5b	1.29 ± 0.10bc	75.85 ± 4.62c	9.18 ± 1.42c	9.9 ± 1.8cd	1202 ± 368c	1665 ± 109ab

Data represent mean ± SD. Means within a column with different letters are statistically different in the element levels between studied sites at $P < 0.05$ for $N = 5$.

The level of physodalic acid significantly increased in all transplanted lichens compared to control thalli from Borecka Forest (Figure 6B). The highest concentrations were in lichens transplanted to Bukowno and Alwernia (63 and 13% higher than in control samples, respectively; Figure 6B). Additionally, increases in the percentage contribution of physodalic acid in the crude extract were observed in samples from Borecka Forest (17.1% increase), in Bukowno (25.8% increase), and in Alwernia (20.6% increase) (Table 2).

Concentrations of atranorin increased in thalli transplanted to Jankowice (by 36%), Bukowno (by 30%), and Młoszowa (by 16%) (Figure 6C). A decrease in atranorin level was observed in lichen samples transplanted to

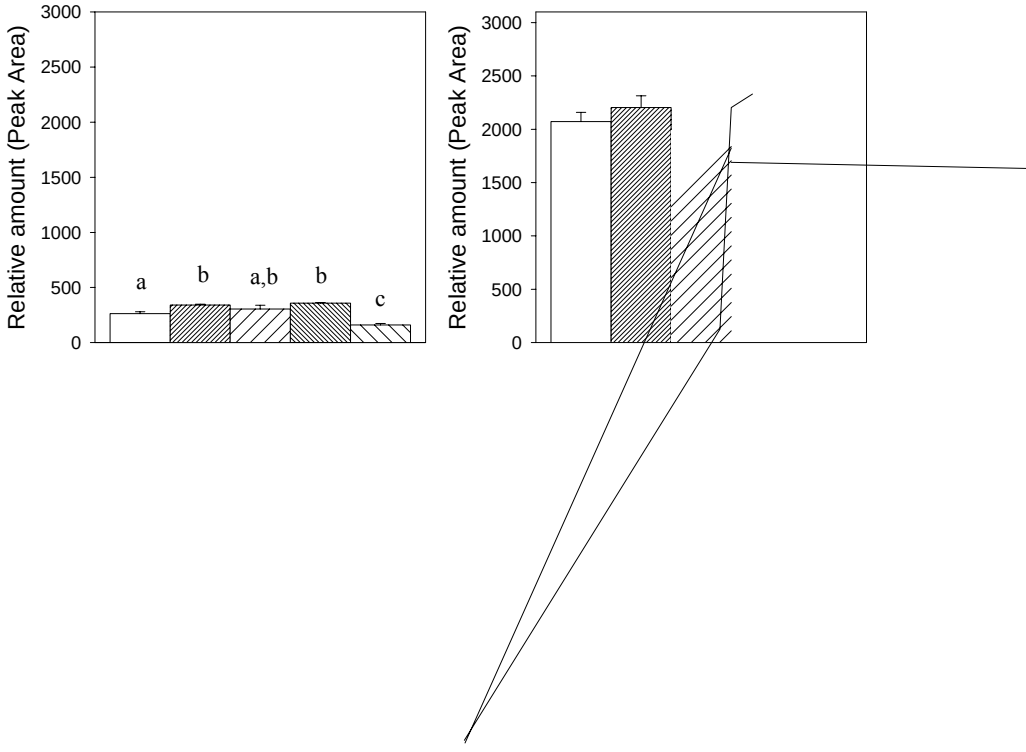

TABLE 2. RELATIVE AMOUNTS (%) OF THE SECONDARY METABOLITES IN THE EXTRACT ISOLATED FROM *H. physodes* FROM VARIOUS TEST LOCATIONS

Location	Unidentified (Rt = 4.4) (%)	Hydroxyphysodic acid (%)	Physodalic acid (%)	Physodic acid (%)	Atranorin (%)
Borecka	–	18.4	17.1	23.5	2.3
Bukowno	–	20.0	25.8	25.8	3.1
Młoszowa	–	18.7	23.7	24.4	2.9
Jankowice	–	21.7	24.4	22.2	3.1
Alwernia	12.1	16.5	20.6	11.5	1.7

Relative peak area was used.

Alwernia. Similar tendencies of this compound were noted on a percentage basis (Figure 6C, Table 2).

An increase in hydroxyphysodic acid concentration was observed in *H. physodes* transplanted to Jankowice forest (Figure 6D). However, the percentage in the crude extract was not changed (Table 2). In lichen transplanted to Alwernia, thalli contained lower levels of hydroxyphysodic acid than control thalli (Figure 6D).

Crude extracts obtained from lichens transplanted to Alwernia contained a lower percentage of physodic acid, hydroxyphysodic acid, and atranorin. Additionally, an unidentified peak (yellow oily compound) with a retention time (Rt) of 4.1 was observed in *H. physodes* samples from Alwernia and constituted 12% of the crude extract (Table 2).

DISCUSSION

Accumulation of elements in transplanted *H. physodes* revealed significant spatial diversification of air pollution in the investigated Cracow–Silesia industrial region. According to our results, sites were classified as either heavily polluted (Bukowno and Alwernia) or moderately polluted (Młoszowa and Jankowice), with less harmful impact of neighbor industries on the environment. Areas with high levels of air pollution are directly influenced by emissions from the Zn–Pb smelter (Bukowno) and from the chemical industry (Alwernia).

To assess whether our results were unusually high, the obtained concentrations of elements were checked against baseline values for chemical elements in *H. physodes*, the most studied lichen in the monitoring of air quality (compiled by Bennett, 2000; Table 3). We found the highest concentrations of Pb, Cd, and Zn in lichens transplanted near the Zn–Pb smelter, where values for these elements were either equal (Pb) or exceeded means for enriched areas

TABLE 3. STATISTICAL BASELINE VALUES FOR CHEMICAL ELEMENTS IN *H. physodes* (AFTER BENNETT, 2000)

	Elements ($\mu g\ g^{-1}$ dry weight)							
	Pb	Zn	Cd	Cr	Ni	Cu	Fe	S
Background average	19.5	73	0.56	2.11	1.72	6.0	621	738
Enriched average	126.9	427	2.56	43.50	12.28	28.6	3081	1695

"Background^—any site not in the city, nor near a point source, and not under any kind of local pollutant influence. "Enriched^—opposite to these criteria.

(Cd, Zn; Table 3). In the rest of the transplantation sites, concentrations of Zn and Pb were equal or lower than mean levels for background areas. Cd was lower than mean levels for enriched areas (Table 3). Baranowska-Bosiacka et al. (2001) found that Pb and Cd accumulated in *H. physodes* remained mainly on the surface of the thalli, whereas Zn and Cu penetrated into the protoplast. Microparticle-induced X-ray emission (PIXE) analysis of thalli transplanted to polluted sites revealed that Zn was concentrated in the algal layer and lower cortex, whereas Cu and Pb were detected only from analyses of smaller areas in individual layers of thalli (Budka et al., 2002). Although Cd was detected with AAS, it was not visible in PIXE studies that confirmed the surface deposition (Budka et al., 2004). We noted the highest levels of Cr and Ni in *Hypogymnia* transplanted near a chemical factory (Alwernia) producing chromium, phosphorus, and sulfur compounds. Cr values were twice the mean for enriched areas, whereas Ni was close to it. In the rest of our test sites, Cr and Ni levels were equal or lower than the mean for background areas (Table 3). Neither Cu nor Fe exceeded threshold levels, even in the most polluted sites (Table 3). PIXE analysis of transplanted *H. physodes* revealed the highest concentrations of Fe in the lower cortex, which suggests a transport mechanism within thalli or a nonatmospheric origin of this element. Additionally, low concentrations of Fe were found in the upper part of the lichen, which could be from dust distributed on the surface (Budka et al., 2004). Especially high values in relation to data given by Bennett (2000) were noted for S levels. Even control lichen from the Borecka forest had almost twice the S levels than the mean for background areas. These levels exceeded the threshold level in the rest of studied sites (Table 3) and reflect the widespread occurrence of air pollution containing sulfur compounds over Poland.

Before the symptoms of different kinds of stress, including pollution, can be visualized by morphological or ultrastructural changes, less obvious results occur on the molecular or chemical level. These less salient markers have become a subject of interest in recent years as they may be used as early indicators of environmental stress on a target organism before damage occurs.

The potential use of secondary metabolites as markers for environmental stress has been postulated for plants and lichens (Jones and Coleman, 1989; Zobel and Nighswander, 1990, 1991; Zobel, 1996; Rabotti and Ballarin-Denti, 1998; Loponen et al., 2001; Caviglia et al., 2001). Moreover, because secondary substances play a significant role in ecosystem interactions, and actions of different kinds of stressors cause modification in their quantity and composition, which, in turn, influences other ecosystem partners (Larcher, 1995). For this reason, secondary compounds can also be used as warning indicators for the surrounding ecosystem (MacGillivray and Helleur, 2001).

Extracts from *H. physodes* contained several biologically active compounds (Huneck and Yoshimura, 1996) and may influence the growth of other organisms in their surroundings. For example, physodic acid has anticancer, antimutagenic, antiviral, and allergenic activities and may inhibit cell division. Atranorin has anticancer, fungitoxic, and antimicrobial activities and may also influence growth of plants as well as growth and development of insects. Hydroxyphysodic acid has potent insecticidal activity (Huneck and Yoshimura, 1996; Hunek, 1999; Romagni and Dayan, 2002). Therefore, it is possible that changes in concentration may influence the fitness of lichens to their environment. Because all of the described metabolites may play a role in the interaction of a lichen with its biotic environment, any reduction in concentration may be reflective of the health of lichens and lower resistance to herbivore attack, diseases, as well as interactions with lower plants (e.g., algae and bryophytes; Huneck, 1999), and host trees. In another study, the metabolites from epiphytic lichen were found in the xylem of the host (Avalos et al., 1986).

The most detrimental changes were observed in secondary metabolites of lichens transplanted to Alwernia, which accumulated high levels of Cr. Significant decreases in hydroxyphysodic acid, physodic acid, and atranorin were observed in thalli collected 6 months after transplantation. Interestingly, crude extracts of lichens transplanted to Alwernia contained an additional unidentified polar peak in chromatograms (Rt = 4.1, denoted as an asterisk in Figure 4) that constituted as much as 12% of the crude extracts. This peak was not observed in lichen extracts from the other sites, including control thalli. After isolation, this polar fraction eluting as a single peak (Figure 4) was a dark yellowish, oily substance and most probably is the product of degradation of lichen acids caused by accumulated chromium ions. According to Jezierski et al. (1999), intensified free radical production in lichens exposed to air pollution is the result of degradation of lichen acids to β-diketones. The unidentified peak might also be a compound produced by lichens for the purpose of defense against stress associated with the accumulation of pollutants in thalli.

A decrease in physodic acid was observed in lichens transplanted to both of the most polluted sites—the forests in Alwernia and Bukowno. In Bukowno, significant accumulations of Pb, Cd, and Zn were noted in thalli collected after

the transplantation period. However, the decrease was more intense in thalli that accumulated Cr, suggesting the reductive power of this element on the degradation of physodic acid.

Thalli of lichens transplanted to all of the test sites in the Silesia–Cracow industrial region contained higher concentrations of physodalic acid than control thalli. Thus, biosynthesis of physodalic acid seems to be elicited in response to stress caused by exposure to heavy metals. Data found in the literature show physodalic acid as an antimutagenic compound (Romagni and Dayan, 2002). There has been no record of detoxifying activity for this substance. The slight increase in atranorin found in lichens transplanted to Bukowno, Młoszowa, and Jankowice suggests a possible role of this compound in detoxification, or it may be a precursor of other phenolic compounds.

In summary, significant, site-specific changes in chemistry of thalli were found in lichens transplanted to the polluted regions selected in this study. The most detrimental alterations were detected in thalli that accumulated high levels of Cr after transplantation near the chemical industry. Decreases in the levels of physodic acid, hydroxyphysodic acid, and atranorin were detected, and one additional peak appeared in the extract. A decrease in physodic acid concentration was also observed in lichens transplanted to the other most highly polluted site near the Zn–Pb smelter as the effect of accumulations of Cd, Pb, and Zn. An increase in the content of physodic acid in all transplanted lichens suggests a role of this compound in defense against stress caused by accumulated pollutants.

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